THERAPEUTIC NANOARTICLES

FIELD OF THE INVENTION

The present invention is directed to the field of therapeutic entities. More specifically, this invention relates to particles with desirable therapeutic attributes containing aminoglycosides.

BACKGROUND OF THE INVENTION

Aminoglycosides, widely used as antibacterial agents, constitute a family of compounds known to bind to oligonucleotides. . Aminoglycosides have also recently shown some promising early results in additional therapeutic applications. For example aminogycosides may enable a read through of a mutant premature termination signal in the gene for cystic fibrosis transmembrane conductance receptor (CFTR). Thus aminoglycosides may allow the treatment of certain genetic disorders, such as cystic fibrosis, or the disruption of the mRNA of target enzymes, such as the thymidylate synthase which plays a critical role in cancer therapy.

However, therapeutic use, whether antibiotic or otherwise, is limited by the *in vivo* toxicity of the aminoglycosides in mammals, in particular nephrotoxicity and ototoxicity. Toxicity occurs when the concentrations necessary to get an efficient therapeutic response at sites of infection also trigger the undesirable side effects in healthy tissue. Several efforts to improve antibiotic performance through their inclusion in nanoparticles have been reported in the literature (Pinto-Alphandary H, et al., 2000, Int J Antimicrob Agents **13**, 155-168). Generally, the structures used have been liposomes and polycyanoacrylate structures. Both of these types of nanoparticles have had limited success for a variety of reasons. In the case of liposomes, stability/antibiotic leakiness and a large size that inhibits access to sites of infection are primary hurdles. With polycyanoacrylate nanoparticles, effective incorporation of hydrophilic antibiotics, such as aminoglycosides, has been problematic. New methods that modify the aminoglycoside biodistribution, residence time *in vivo*, solubility, or bioavailability are desirable.

SUMMARY OF THE INVENTION

The present invention is directed to novel hybrid nanostructures that improve the performance and therapeutic index of aminoglycoside antibiotics. More particularly, the invention is directed to hydrophilic polymeric nanoarticles comprising one or more types of

aminoglycoside, aminoglycoside derivative or aminoglycoside-conjugate molecules (all of which are encompassed herein and in the appended claims under the term "aminoglycosides"). The aminoglycosides are in intimate relationship to a polymeric scaffold core comprising hydrophilic building blocks. In a presently preferred embodiment, at least some of the aminoglycosides act as building blocks and are incorporated into the nanoarticle scaffold structure.

Preferably, such articles are easy to fabricate, are biocompatible, are metabolized to nontoxic substances in the body, and are non-immunogenic. Preferred nanoarticles incorporate multiple aminoglycoside molecules. The number of aminoglycoside molecules per nanoparticle may range from 2 to 1000, more preferably from 10 to 1000. The articles of this invention are biodegradable and preferably carbohydrate-based, with the rate of degradation controlled in part by the identity of the carbohydrate, the identity of the saccharide repeats, hydrolyzable or otherwise degradable functionalities present in the carbohydrate chains or in linkages connecting the carbohydrate chains to one another or to the aminoglycoside molecules, the crosslink density, and the presence and distribution of ionic species. Thus, the articles can be metabolized *in vivo*, preventing undesirable accumulation in the body. The articles can be administered to patients by methods generally known in the art. In a presently preferred embodiment, administration is by injection (subcutaneous, intravenous, intramuscular, intradermal, intraperitoneal, intracerebral, or parenteral). The articles may also be suitable for nasal, pulmonary, vaginal, ocular delivery and oral ingestion or released from a depot-form matrix. The articles may also be suspended in a pharmaceutically acceptable carrier for administration.

For applications in which the articles are intended to circulate in the bloodstream of an organism, the articles of the invention are preferably from about 5 nm to about 100 nm in diameter, more preferably from about 5 nm to about 50 nm. The size of the articles allows their use as bioactive entities in mammals. To avoid uptake by the RES system, articles are preferably less than 100 nm. To avoid renal clearance, articles are preferably larger than 5 nm. Reverse microemulsion polymerization can yield article scaffolds of the invention.

The articles of the present invention may be further comprised of recognition elements (REs) to facilitate targeting and/or delivery. By functionalizing the nanoarticles with ligands that target either markers of the infected tissue or directly the pathogen, the local antibacterial concentration of the aminoglycoside in the proximity of the infectious agent can be increased to levels where the chances of selection for bacterial resistance become much less likely. At the same time, localization of aminoglycosides away from the sites prone to tissue damage, namely the kidneys and the inner ear, will limit side effects, thereby increasing the therapeutic margin of aminoglycoside antibiotics. Targeting could

also allow treatment for humans presenting a point mutation in the 125 rRNA gene that causes an aminoglycoside hypersensitivity (Hutchin T. & Cortopassi G., 1994, Antimicrob. Agents Chemother. **38**, 2517-2520). For gene silencing applications, the targeting can provide specific accumulation of aminoglycosides in the affected organ(s).

The articles may also optionally be comprised of surface-attached polymers that can serve as linkers or tethers with one end attached to the article surface and the other end functionalized with a recognition element. Useful surface attached polymers may be comprised of polyethylene glycol (PEG), poly(glutamate), and/or polysaccharides. The surface-attached polymers may provide other useful or desirable characteristics to the hydrophilic articles, such as for example enhanced circulation.

The invention is further directed to methods of synthesizing the aminoglycoside-containing nanoarticles of the invention. The article scaffolds are formed in the dispersed aqueous phase of a reverse (water in oil) microemulsion. Modular assembly of water-soluble building blocks in a reverse microemulsion provides a flexible platform to construct AG nanoarticles. By varying the building block composition, reactive moieties can be incorporated in the nanoarticle architecture, which provide attachment points for recognition elements through orthogonal chemistry.

The invention is also directed to the various applications for which the aminoglycoside-containing nanoarticles of the invention may be used. The present invention provides a method of improving the activity of aminoglycosides by incorporating the AGs in intimate relationship to a less positively-charged, preferably neutral or negatively charged polymeric nanostructure. By "improved activity" is meant that the AG-containing nanoarticles of the invention exhibit at least one of the following: greater therapeutic activity, longer retention time in the body, and fewer or no side effects, such as lowered bacterial resistance and/or decreased toxicity (particularly nephrotoxicity and ototoxicity). The AG-containing nanoarticles have a high efficacy with low toxicity in comparison to AGs alone. The aminoglycoside-containing nanoarticles may be used for the treatment of not only aerobic, Gram-negative bacterial infections but also of nonaerobic, Gram-positive bacterial infections, while reducing side effects, including toxicity. The hydrophilicity, the size, the charge at physiological pH, and the method of aminoglycoside incorporation, as well as the optional use of recognition elements, are all designed to concentrate the antibiotic to the site of infection. The articles can also be used in in vitro experiments to help elucidate the mechanisms by which aminoglycosides reach the bacterial cytoplasm and their biomolecular targets.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an illustration of the structure of an example of an aminoglycoside-containing hydrogel nanoarticle of the invention, where "AG" represents an aminoglycoside.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "a" and "an" mean "one or more", unless otherwise indicated.

As used herein, the terms "articles" and "nanoarticles" are used interchangeably.

By "water-soluble" is meant, herein, having a solubility in water of greater that 10 mg/mL, and preferably greater than 50 mg/mL.

As used herein, "building blocks" means the molecular structures that are brought together through physical and chemical bonds to form the hydrogel nanoparticle.

We adopt herein a source-based nomenclature and description of the nanoarticles disclosed in this invention. That is, we follow IUPAC guidelines which specify the naming of polymer structures based upon the molecules (the source) that were reacted together to form the polymer. Thus, when we disclose that a nanoarticle is comprised of certain building blocks, we mean that those building block molecules were reacted together to form the scaffold. For example, if we specify that a nanoarticle is comprised of a carbohydrate, we mean that a carbohydrate was reacted with other molecules to form the nanoarticle. In another example, if we specifiy an "AG-nanoarticle" we mean that AG molecules were reacted with other building blocks that comprise the nanoarticle.

As used herein, the terms "nanoarticle scaffold", "article scaffold", "hydrogel scaffold" and "scaffold" are used interchangeably and refer to a crosslinked, nanoscopic hydrogel structure that does not include tether molecules or recognition elements. The scaffold may or may not be formed in part of aminoglycoside or aminoglycoside derivative building blocks.

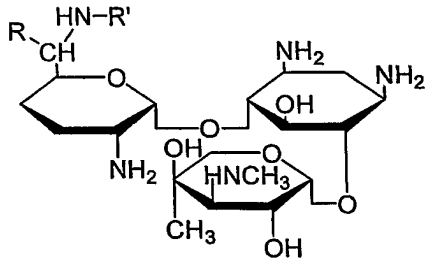
The terms "recognition elements" or "REs" are used herein to define structures that bind to biomolecular targets found in mammals or on bacteria or other microorganism. In the present invention, the REs may target either the pathogen or the site of infection. The number of REs per aminoglycoside-containing nanoarticle can range from 2 to about 1000, preferably from 2 to 500. The articles may optionally further be comprised of more than one type of RE. As used herein, a RE "type" is defined as an RE of a specific molecular structure. An additional advantage of the present invention is that multiple RE types with complementary features may be incorporated into a single article.

The nanoarticles of the invention comprise aminoglycosides and a hydrogel scaffold core, wherein the aminoglycosides are in "intimate relationship" to the article scaffold core; that is, they are either physically or chemically, or both physically and chemically, incorporated into or onto the scaffold. The scaffold is formed of crosslinked hydrophilic

building blocks. The articles may further optionally comprise recognition elements and/or surface-attached polymers. The articles have reduced positive charge compared to the charge/mass of constituent aminoglycoside molecules alone, and may have an overall neutral or negative charge. The articles are hydrophilic and intended for use in mammals. The invention is further directed to methods of synthesizing these polymeric articles.

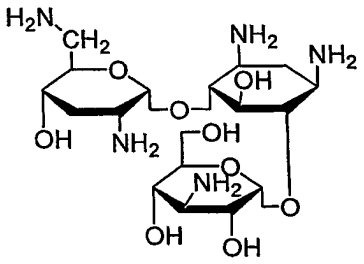
The articles of the invention may range in size from about 5 nm to about 1000 nm, more preferably from about 5 nm to about 100 nm in diameter, and most preferably about 10 nm to about 70 nm. Articles in the 10 to 70 nm size range may effectively avoid both renal clearance and uptake by the reticuloendothelial (RES) system. Additionally, such articles may advantageously exit the blood stream to reach sites of infection, infectious microorganisms or other desired cells, tissues, and organs.

Aminoglycosides are a group of similar molecules that have demonstrated antibiotic activity. Aminoglycosides include at least eight drugs: amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin, and tobramycin. Representative molecular structures are presented below. Formula A is gentamicin, Formula B is tobramycin, and Formula C is amikacin.

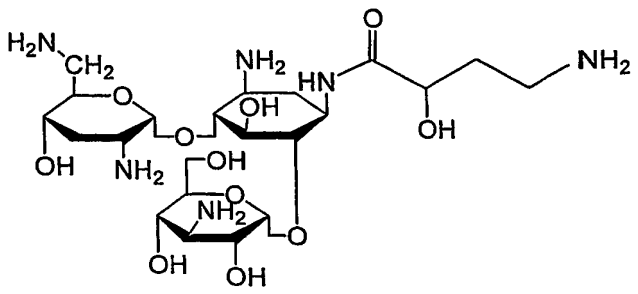


Formula A

R=R'=CH_3
R=R'=H
R=CH_3, R'=H



Formula B



Formula C

For convenience, aminoglycosides and aminoglycoside chemical structures may be indicated generically herein in the text by the designation "AG" and in the chemical structures as "AG-$(NH_2)_p$", where "NH_2" indicates the free amine groups pendant on the aminoglycoside (AG) and "p" is the number of such pendant groups, normally from 1 to 7 depending upon the particular aminoglycoside.

Aminoglycosides are highly flexible molecules and, although not shown in the chemical formulas, at physiological pH they have multiple protonated amino groups, and thus are positively charged. The positive charges are attracted to the negatively charged backbone of RNA and bind to certain RNA elements, the resulting binding leading to inhibition of protein biosynthesis. However, the positive charges reduce the therapeutic index of aminoglycosides due to fast pharmacokinetic clearance and limited biodistribution, along with causing significant toxicity. In particular, because aminoglycosides are polycations at physiological pH, they cross membranes very poorly, do not penetrate the CNS or the eye, and are easily taken up by proximal tubular cells, all of which account for the narrow margin of their therapeutic index.

Incorporation of various amounts of negatively or positively charged building blocks in the AG-containing nanoarticle construct allows the fine-tuning of the overall charge on the article. Article charge, which can be characterized by Zeta potential measurements, can have influence on the mechanisms of uptake and adhesion. In contrast to the positively charged AGs, the AG-containing nanoarticles of the present invention are synthesized with a reduced positive charge compared to the charge/mass of constituent aminoglycoside molecules alone, and may have an overall neutral or slightly negative charge. Mostly because of their cationic polar nature, AGs such as tobramycin do not readily cross the membranes of epithelial cells (Neu HCC (1976) *J. Infect. Dis*, Suppl. **134**, S3-14 in Physician Desk Reference, TOBI, 2004 Ed., p 1170) in which *P. aeruginosa* accumulates, for example at the site of lung or corneal infections. On the other hand, our studies have shown the nanoarticles to be easily internalized by epithelial and various other kinds of cells. While the nanoarticle can facilitate the delivery of AG into the epithelial cells of the host, the overall

negative or neutral charge of the AG nanoarticle allows for a longer circulation time and less uptake by RES. The size of the nanoarticle above glomerular filtration also accounts for reduced kidney clearance. In the case of targeted AG-containing nanoarticles, a longer circulation time will favor recognition over non-specific binding.

Hydrophilic building blocks with polymerizable groups are employed to form the hydrogel scaffold. In one embodiment of the invention, the nanoarticles are formed by self-assembly of the building blocks through ionic, Van der Waals, and or hydrogen bond interactions. Examples of such articles include, but are not limited to, carbohydrates able to form physical (that is, not covalently crosslinked) gels, such as mixtures containing pectins or alginates.

In another embodiment of the invention, building blocks have reactive groups that are crosslinked in the dispersed aqueous phase of reverse microemulsions. The number of polymerizable groups attached to one single building block can range, for example, from about one to three for low molecular weight building blocks, to ten or more for polymeric building blocks. Building blocks that contain more than one reactive group can act as crosslinking agents and enable the formation of a hydrogel scaffold. Using different amounts and proportions of building blocks from a set of building blocks with one, two, or more reactive groups allows formation of hydrogels of different compliancy upon polymerization.

Article Scaffold Fabrication in Reverse Microemulsions: Articles of the present invention can be fabricated by first forming nanoscopic hydrogel scaffolds through the crosslinking of hydrophilic building blocks solubilized in the dispersed water phase of a reverse microemulsion. The organic solvent and non-reactive surfactants are removed after polymerization to yield crosslinked, water-soluble nanoscopic articles. The building blocks include the "scaffold building blocks", which crosslink with each other and, in some embodiments, with the AGs to provide the primary structure to the nanoarticles. "Crosslinkable building blocks" may also be included, to provide further crosslinking between the scaffold building blocks. In certain embodiments, at least some of the aminoglycosides are chemically incorporated with the scaffold building blocks into the nanoarticle scaffold core structure and thus function as building blocks as well.

Reverse microemulsions for scaffold fabrication are formed by combining aqueous buffer or water, building blocks, organic solvent, surfactants and initiators in the appropriate ratios to yield a stable phase of surfactant-stabilized aqueous nanodroplets dispersed in a continuous oil phase. Stable reverse microemulsion formulations can be found using known methods by those skilled in the art. They are discussed, for example, in *Microemulsion Systems*, edited by H. L. Rosano and M. Clausse, New York, N.Y.: M. Dekker, 1987; and in

Handbook of Microemulsion Science and Technology, edited by P. Kumar and K.L. Mittel, New York, N.Y.: M. Dekker, 1999. In this invention, an aqueous phase with solubilized hydrophilic building blocks is added to an organic solvent containing one or more solubilized surfactants to form a reverse microemulsion.

The dispersed aqueous phase contains hydrophilic building blocks solubilized at from about 5 to about 65 wt%, preferably from about 5 to about 25 wt%. While not wishing to be bound by theory, the use of high water-content hydrogel scaffolds also may reduce immunogenicity in end uses, because there is less foreign surface for immune system components to recognize. The high water content also provides compliancy through a more flexible scaffold. Thus, when attaching to cell surface receptors, the articles are able to conform to the cell surface, allowing more surface receptors to be bound. Binding more receptors may allow the article to better function as an antagonist. Additionally, while not wishing to be bound by theory, it is believed that article cell surface coverage can inhibit other cell signaling pathways.

Polymerization of the building blocks in the nanodroplets of the dispersed aqueous phase of the reverse microemulsion follows procedures known to those skilled in the art (see, for example, G.G. Odian, *Principles of Polymerization*, 3rd Ed., Wiley, New York, 1991; L.H. Sperling, *Introduction to Physical Polymer Science,* Chapter 1, pp. 1-21, John Wiley and Sons, New York, 1986; and R.B. Seymour and C.E. Carraher, *Polymer Chemistry*, Chapters 7-11, pp. 193-356, Dekker, New York, 1981). Polymerization has been performed in the dispersed phase of microemulsions and reverse microemulsions (for a review, see Antonietti, M.; and Basten, R., Macromol. Chem. Phys., 1995, *196*, 441; for a study of the polymerization of a hydrophilic monomer in the dispersed aqueous phase of a reverse microemulsion, see Holtzscherer, C. and Candau, F., Colloids and Surfaces, 1988, *29*, 411). Such polymerization can yield articles in the 5 nm to 50 nm size range.

The size of the nanodroplets of the dispersed aqueous phase is determined by the relative amounts of water, surfactant and oil phases employed. Surfactants are utilized to stabilize the reverse microemulsion. These surfactants do not include crosslinkable moieties; they are not building blocks. Surfactants that may be used include commercially available surfactants such as Aerosol OT (AOT), polyethyleneoxy(n)nonylphenol (Igepal™, Rhodia Inc. Surfactants and Specialties, Cranbrook, NJ), sorbitan esters including sorbitan monooleate (Span® 80), sorbitan monolaurate (Span® 20), sorbitan monopalmitate (Span® 40), sorbitan monostearate (Span® 60), sorbitan trioleate (Span® 85), and sorbitan tristearate (Span® 65), which are available, for example, from Sigma (St Louis, MO). Sorbitan sesquioleate (Span® 83) is available from Aldrich Chemical Co., Inc. (Milwaukee, WI). Other surfactants that may be used include polyoxyethylenesorbitan (Tween®)

compounds. Exemplary cosurfactants include polyoxyethylenesorbitan monolaurate (Tween® 20 and Tween® 21), polyoxyethylenesorbitan monooleate (Tween® 80 and Tween® 80R), polyoxyethylenesorbitan monopalmitate (Tween® 40), polyoxyethylenesorbitan monostearate (Tween® 60 and Tween® 61), polyoxyethylenesorbitan trioleate (Tween® 85), and polyoxyethylenesorbitan tristearate (Tween® 65), which are available, for example, from Sigma (St Louis, MO). Other exemplary commercially available surfactants include polyethyleneoxy(40)-sorbitol hexaoleate ester (Atlas G-1086, ICI Specialties, Wilmington DE), hexadecyltrimethylammonium bromide (CTAB, Aldrich), and linear alkylbenzene sulfonates (LAS, Ashland Chemical Co., Columbus, OH).

Other exemplary surfactants include fatty acid soaps, alkyl phosphates and dialkylphosphates, alkyl sulfates, alkyl sulfonates, primary amine salts, secondary amine salts, tertiary amine salts, quaternary amine salts, n-alkyl xanthates, n-alkyl ethoxylated sulfates, dialkyl sulfosuccinate salts, n-alkyl dimethyl betaines, n-alkyl phenyl polyoxyethylene ethers, n-alkyl polyoxyethylene ethers, sorbitan esters, polyethyleneoxy sorbitan esters, sorbitol esters and polyethyleneoxy sorbitol esters.

Other surfactants include lipids, such as phospholipids, glycolipids, cholesterol and cholesterol derivatives. Exemplary lipids include fatty acids or molecules comprising fatty acids, wherein the fatty acids include, for example, palmitate, oleate, laurate, myristate, stearate, arachidate, behenate, lignocerate, palmitoleate, linoleate, linolenate, and arachidonate, and salts thereof such as sodium salts. The fatty acids may be modified, for example, by conversion of the acid functionality to a sulfonate by a coupling reaction to a small molecule containing that moiety, or by other functional group conversions known to those skilled in the art.

Additionally, polyvinyl alcohol (PVA), polyvinylpirolidone (PVP), starch and their derivatives may find use as surfactants in the present invention.

Cationic lipids may be used as cosurfactants, such as cetyl trimethylammonium bromide/chloride (CTAB/CTAC), dioctadecyl dimethyl ammonium bromide/chloride (DODAB/DODAC), 1,2-diacyl-3-trimethylammonium propane (DOTAP), 1,2-diacyl-3-dimethyl ammonium propane (DODAP), [2,3-bis(oleoyl)propyl] trimethyl ammonium chloride (DOTMA), and [N-(N'-dimethylaminoethane)-carbamoyl]cholesterol, dioleoyl) (DC-Chol). Alcohols may also be used as cosurfactants, such as propanol, butanol, pentanol, hexanol, heptanol and octanol. Other alcohols with longer carbon chains may also be used.

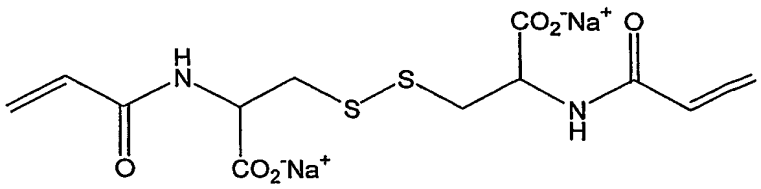
In one preferred embodiment of the invention, the scaffold is crosslinked by **Free-Radical Polymerization:** Preferred polymerizable functionalities are acrylate, acrylamide, methacrylate, and methacrylamide moieties. Such moieties are amenable to free-radical polymerization. Free-radical polymerization can be readily achieved through the combination

of UV and/or visible light and photoinitiators, redox-coupled free-radical initiators, or heat and heat-activated initiators.

Building blocks that may be used to form the article scaffold include small molecule building blocks with one reactive group or multiple reactive moieties that can act as crosslinkers in the hydrogel scaffold. Exemplary reactive groups include, but are not limited to, acrylate, acrylamide, vinyl ether, styryl, epoxide, maleic acid derivative, diene, substituted diene, thiol, alcohol, amine, hydroxyamine, carboxylic acid, carboxylic anhydride, carboxylic acid halide, carboxylic acid hydrazide, hydrazide, aldehyde, benzaldehyde, ketone, isocyanate, succinimide, hydrazine, thiohydrazide, glycidyl ether, siloxane, alkoxysilane, alkyne, azide, 2'-pyridyldithiol, phenylglyoxal, iodo, maleimide, imidoester, dibromopropionate, bromoacetyl, and iodacetyl moieties.

Exemplary building blocks include acrylamide, sodium acrylate, diacetone acrylamide (DAA), levulinic acid acrylamide, methylene bisacrylamide, ammonium 2,2-bisacrylamidoacetate, 2-acrylamidoglycolic acid, 2-aminoethyl methacrylate, aminopropyl methacrylate (APMA), ornithine mono-acrylamide, ornithine diacrylamide sodium salt, N-acryloyltris(hydroxymethyl)-methylamine, N-methacryloylated short peptides made according to US 5,037,883, hydroxyethylacrylate, N-(2-hydroxypropyl)-acrylamide, 2-sulfoethylmeth-acrylate, 2-methacryloylethyl glucoside, glucose monoacrylate, glucose-1-(N-methyl)-acrylamide, glucose-2-acrylamide, glucose-1,2-diacrylamide, maltose-1-acrylamide, sorbitol monoacrylate, sorbitol diacrylate, sucrose diacrylate, sucrose mono(ethylenediamine acrylamide), sucrose di(ethylenediamine acrylamide), sucrose di(diethylenetriamine acrylamide), kanamycin tetraacrylamide, kanamycin diacrylamide, sucrose mono(ethylenediamine acrylamide) mono(diethylenetriamine acrylamide) mono(phenyl alanine) sodium salt, as well as other acrylate- or acrylamide-derivatized sugars.

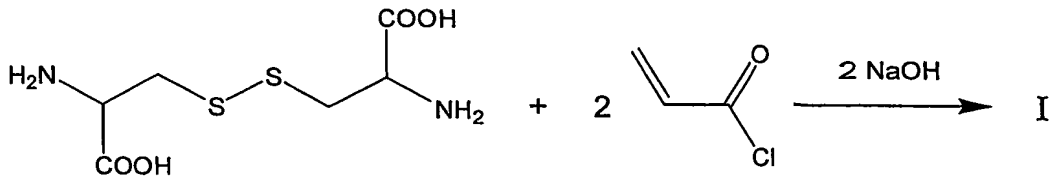
Building blocks are chosen to achieve a desired content of certain functionalities in the article scaffold. Such functionalities can improve solubility and may also be used as points of attachment for REs or aminoglycosides. In a preferred embodiment, at least some of the building blocks are a cystine bis-acrylamide (CiBA) monomer, which has the following formula I:



I

CiBA may be prepared by reacting L-cystine (II, a derivative of the amino acid cysteine) with 2 equivalents of acryloyl chloride (III), according to the following reaction Scheme 1:

Scheme 1: Synthesis of CiBA



II III

CiBA is water-soluble and is capable of polymerizing with other building blocks to form the polymeric nanoarticle core. In addition, its disulfide linkage provides, after reduction, free thiols for attachment of REs to the core structure.

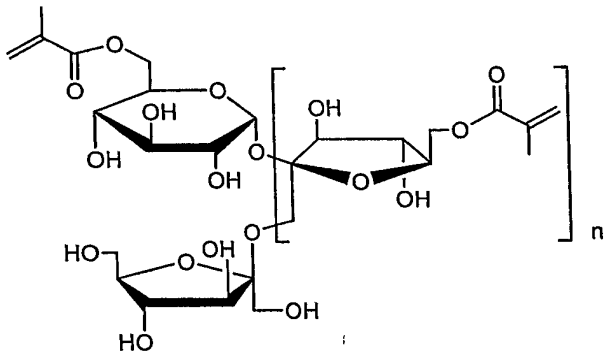
In a preferred embodiment, at least some of the building blocks are carbohydrates. In the case of carbohydrate building blocks, the carbohydrate region is usually comprised of a plurality of hydroxyl groups, wherein at least one hydroxyl group is modified to include at least one polymerizable group. The carbohydrate region of the carbohydrate building block may include a carbohydrate or carbohydrate derivative. For example, the carbohydrate region may be derived from a simple sugar, such as glucose, ribose, arabinose, xylose, lyxose, allose, altrose, mannose, gulose, idose, galactose, fructose, malonate, syalic acid or talose; a disaccharide, such as maltose, sucrose or lactose; a trisaccharide; a polysaccharide, such as cellulose, starch, glycogen, alginates, inulin, and dextran; or modified polysaccharides. Other representative carbohydrates include sorbitan, sorbitol, chitosan and glucosamine. The carbohydrate may include amine groups in addition to hydroxyl groups, and the amine or hydroxyl groups can be modified, or replaced, to include a crosslinking group, other functionalities, or combinations thereof.

Carbohydrate-based building blocks may be prepared from the carbohydrate precursor (e.g. inulin, dextran, polygalacturonic acid, starch derivatives, etc.) by standard coupling technologies known in the art of bioorganic chemistry (see, for example, G Hermanson, *Bioconjugation Techniques*, Academic Press, San Diego, 1996, pp 27-40, 155, 183-185, 615-617; and S. Hanesian, *Preparative Carbohydrate Chemistry*, Marcel Dekker, New York, 1997.) For example, a crosslinkable group is readily attached to a carbohydrate via the dropwise addition of acryloyl chloride to an amine-functionalized sugar. Amine-functionalized sugars can be prepared by the reaction of ethylene diamine (or other amines)

with 1,1'-carbonyldiimidazole-activated sugars. Other reactions that introduce an amine on the carbohydrate may also be used, many of which are outlined in *Bioconjugation Techniques (supra)*. Ester-linked reactive groups can be synthesized through the reaction of acrylic or methacrylic anhydrides with the hydroxyl group of a carbohydrate such as inulin in pyridine.

Carbohydrate-based building blocks may also be prepared by the partial (or complete) functionalization of the carbohydrate with moieties that are known to polymerize under free radical conditions. For example, methacrylic esters may be placed on a carbohydrate at varying substitution levels by the reaction of the carbohydrate with methacrylic anhydride or glycidyl methacrylate (Vervoort, L.et al. International Journal of Pharmaceutics, 1998, *172*, 127-135).

In a presently preferred embodiment, at least some of the building blocks are inulin multi-methacrylate (IMMA) monomer, which has the following formula IV (where n = 1 -100:



Formula IV

The extent to which inulin is functionalized with methacrylate moieties, that is, the number of hydroxyl moieties on inulin that are converted to methacrylic esters to produce IMMA, is a statistical process governed by the concentrations and weight ratios of inulin and methacrylic anhydride starting material. The extent of functionalization may range from one methacrylate for every 1 to 100 monosaccharide repeat units, more preferably one methacrylate for every 3 to 20 monosaccharide repeat units. IMMA can be synthesized with varying degrees of substitution to allow tuning of hydrogel degradation characteristics; the ester linkage to inulin may advantageously function as a site of degradation *in vivo*, allowing the article to degrade and be cleared from the body.

Dextran multiacrylamide and pullulan multiacrylamide are additional preferred building blocks that may be prepared using similar methods.

Carbohydrate-based building blocks may also be prepared by chemoenzymatic methods (Martin, B. D. *et. al.* Macromolecules, 1992, 25, 7081), for example in which *Pseudomonas cepacia* catalyzes the transesterification of monosaccharides with vinyl acrylate in pyridine or by the direct addition of an acrylate (Piletsky, S., Andersson, H., Nicholls, Macromolecules, 1999, *32*, 633-636). Other functional groups may be present, as numerous derivatized carbohydrates are known to those familiar with the art of carbohydrate chemistry.

Besides carbohydrate-based building blocks, other examples of acrylate- or acrylamide-derivatized polymeric building blocks include polyethylene glycol-based molecules, such as polyethyleneglycol diacrylate, with molecular weights ranging from 200 to 40,000 daltons.

In a preferred embodiment, to facilitate metabolism of the scaffold and thereby drug release in a desired time frame, degradable covalent linkages are included within the crosslinked nanoarticle core. Similarly, degradable linkages can be included through the use of polylactide, polyglycolide, poly(lactide-co-glycolide), polyphosphazine, polyposphate, polycarbonate, polyamino acid, polyanhydride, and polyorthoester – based building blocks, among others. Hydrolyzable moieties such as carbonates, esters, imines, urethanes, orthoesters, amides, and phosphates may be incorporated as building blocks. To function as degradable components in the nanoarticle core, these building blocks must be functionalized with two or more polymerizable moieties. For example, polyglycolide diacrylate, polyorthoester diacrylate and acrylate-substituted polyphosphazine, acrylate-substituted polyamino acids, or acrylate-substituted polyphosphate polymers can be used as degradable building blocks. Methacrylate or acrylamide moieties can be employed instead of acrylate moieties in the above examples. Similarly, small molecules containing a hydrolyzable segment and two or more acrylates, methacrylates, or acrylamides may be used. Such degradable polymers and small molecule building blocks may be functionalized with acrylate, methacrylate, acrylamide or similar moieties by methods known in the art.

Incorporation of Aminoglycosides:

Aminoglycosides can be incorporated in the article in several ways, including:

i) The aminoglycosides can be linked to a preformed hydrogel scaffold via physical interactions such as hydrogen bonds, ionic or van der Waals interactions.

ii) The aminoglycosides can be linked to a preformed hydrogel scaffold by covalent bonds.

iii) The aminoglycosides can be physically entrapped during the formation of the scaffold.

iv) The aminoglycosides can act as one of the building blocks used to form the scaffold via chemoselective polymerization.

v) The aminoglycosides can be pre-assembled with or functionalized into polymerizable building blocks that are polymerized into the scaffold by methods known of the art, preferentially by free radical polymerizations.

Where aminoglycosides are linked to a preformed hydrogel scaffold, it is preferable to functionalize the articles in an aqueous or partially aqueous system. More preferentially, the aminoglycoside molecules are covalently linked to the article scaffold. The aminoglycosides can be linked either directly or through a linker molecule (surface-attached polymer) to the nanoarticle, which must contain a functionality that allows attachment. Preferably, although not necessarily, this functionality is one member of a pair of chemoselective reagents selected to aid the coupling reaction. (Lemieux, G., Bertozzi, C., *Trends in Biotechnology*, 1998, *16*, 506-513). For example, many polymerizable building blocks contain acidic moieties, which are accessible at the surface of the articles after their polymerization; coupling of the carboxylic functions, for example with EDC and NHS, is then possible with the amines on the aminoglycosides. Alternatively, the aminoglycosides can be reacted with the carboxylate terminus of a heterofunctionalized PEG chain that presents a function such as a thiol or a haloacetamide at the other extremity.

In another example, the article core surface (and/or linkers grafted to its surface) displays a ketone or an aldehyde moiety, allowing formation of Schiff base attachment points.

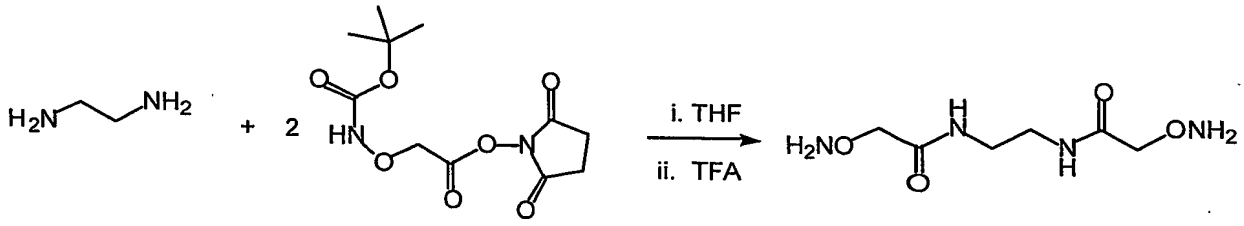
It is possible to functionalize the articles with several coupling strategies, varying both the order of addition of the different components and the reactive chemical moieties used for the coupling. Methodologies are known or can be determined by those of ordinary skill in the art without undue experimentation (see, for example, International PCT Publn. WO 03/101425, the disclosure of which is incorporated by reference herein).

In another embodiment of the invention, the aminoglycosides are physically incorporated into the nanoarticle during the formation of the article. The AGs are placed into the water phase of the reverse microemulsion together with building blocks, and the nanoarticle scaffold is formed at least in part by chemoselective polymerization of the building blocks, entrapping the AGs. A representative example of this strategy may be the use of dithiol compounds, such as PEG-dithiol, dithiothreitol, short cysteine-containing peptides, or thiol-derivatized carbohydrates, obtained for example by addition of Traut's reagent to amine-containing carbohydrates on one hand, with building blocks containing active halogens, such as bromoacetamide or iodoacetamide, or maleimide moieties on the other hand. Many difunctional or trifunctional cross-linking reagents are available from

commercial sources such as Pierce (www.piercenet.com) to introduce haloacetal, maleimide, imidoester or NHs-esters on alcohol, amine, acid or thiol containing building blocks.

In another method, a polysaccharide may be partially oxidized to contain numerous aldehydes. Alternatively, a mixture containing glutaraldehyde is used. Compounds containing at least two reactive moieties such as hydrazine, hydrazide, thiocarbazide and hemicarbazide can be used as hydrogel crosslinking agents via reaction with the aldehydes or their hydrazone or carbohydrazone-like derivatives. A di(amino-oxy)-containing compound, such as that made from reacting ethylene diamine with the NHS ester of Boc-amino-oxyacetic acid (see Scheme 2), can also be used as a crosslinking agent through the reaction of the aldehydes of the oxidized sugar reacting with the amino-oxy functionalities.

Scheme 2: synthesis of a di(amino-oxy) crosslinker



Polyamides, such as polyethyleneimine (PEI), spermine, spermidine, 1,3-propan-2-ol, or amine-containing carbohydrates can also be reacted with the aldehydes-containing building blocks to form multiple Schiff bases or imines linkages. In such cases, all or part of the amines from the aminoglycoside are also expected to react with some of the building blocks.

Likewise, in the following chemoselective crosslinking scheme, the aminoglycoside will also react with some of the building blocks: polysaccharides or amines-containing polysaccharides on one hand and di-isocyanides or di-isothiocyanates on the other hand, which react with the hydroxyl and amine moieties of the carbohydrates, can be combined to form, respectively, carbamate or thiocarbamate and isourea or isothiourea functions.

Preferentially, the aminoglycosides are used as the amine-containing moieties in the above chemoselective polymerization methods. They can constitute one of the crosslinking agents as their multiple amine moieties can form ionic bonds, reversible Schiff base formation or be involved in covalent links such as ester and imidoester, carbamate, thiocarbamate, amide, urea or thiourea bonds. In a preferred embodiment, the amine moieties of aminoglycosides or, alternatively, the guanidine moieties such as found in streptomycin, can be reacted with aldehydes- or ketones-containing building blocks and

polymerized in basic pH buffers to form Schiff bases. Examples of such building blocks containing aldehydes or ketones include, but are not limited to, peptide-containing repeats of levulinic acid, glutaraldehyde, oxidized mono- or polysaccharides and their derivatives (such as oxidized inulin, oxidized dextran, oxidized starch, oxidized chitosan), and p-benzaldehyde-dextran and other p-benzaldehyde derivatives of carbohydrates. The different Schiff bases formed can exhibit different release rates. In particular, the benzaldehyde derivatives tend to constitute sturdier bonds which account for higher aminoglycoside incorporation rates and/or slower drug release profiles.

In one preferred embodiment of the invention, the nanoarticle scaffold is formed at least in part by free radical polymerization. Aminoglycosides can be added to the solution of building blocks before free radical polymerization. For physical incorporation, a mixture containing building blocks with acid functions, such as sodium acrylate or CIBA, offers ionic interactions. Aminoglycoside-acrylate salts can be prepared before incorporation into the reverse microemulsion by counterion exchange on a strong anion exchange resin; they are then copolymerized with hydrophilic building blocks in the reverse microemulsion. In another embodiment of the invention, the amine moieties of aminoglycosides can be reacted to form a polymerizable polyamide-glycoside derivative, for example acrylamides. One exemplary method is coupling with a polymerizable building block containing an acid function, with EDC, NHS in aqueous media. Another exemplary method is by reaction with acryloic chloride, methacryloic chloride or acrylic anhydride in suitable solvents. This last method can yield polyacrylamido-glycoside derivatives with varying levels of acrylamide functionalizations, such as but not limited to, kanamycin tetramethacrylate, gentamicin polyacrylamides, tobramycin mono acrylamide, tobramycin bisacrylamide, neomycin monomethacrylamide, neomycin bisacrylamide, neomycin tertaacrylamide, paromomycin monomethacrylamide, paromycin pentacrylate, amikacin monoacrylamide, amikacin tetracrylamide, netilmicin monoacrylamide, or netilmicin bisacrylamide. Such polymerizable building blocks can be polymerized alone or in combination one with another and with the previously mentioned building blocks.

One preferred exemplary method is to pre-assemble the aminoglycosides with building blocks containing aldehydes or ketone functions and moieties polymerizable by free radical polymerization, to allow for Schiff base formation and then to initiate the free radical polymerization of the building-blocks mixture (see FIG. 1). Examples of suitable building blocks include, but are not limited to di-acetone acrylamide (DAA), levulinic methacrylate, multimethacrylate derivatives of oxidized carbohydrates (such as inulin, dextran, starch or pectin), and acrylated, methacrylated or vinyl derivatives of p-benzaldehyde-functionalized carbohydrates such as previously described. The Schiff base bond is reversible and thus

allows the release of the AG from the hydrogel article under appropriate conditions. The bond is particularly well-suited to the incorporation of AGs due to the multiple amines in the AG structure.

Article Functionalization with Recognition Elements:

For applications where it is beneficial to target the nanoarticles, they should present one or more targeting moieties or recognition elements (both of which terms are used interchangeably and are encompassed herein and in the appended claims by the terms "recognition elements" or "REs") to facilitate targeting and/or delivery. The REs may target either the pathogen or the site of infection. The number of REs per article can range from 2 to about 1000, preferably from 2 to 500. The articles may optionally further be comprised of more than one type of RE. As used herein, a RE "type" is defined as an RE of a specific molecular structure. An additional advantage of the present invention is that multiple RE types with complementary features may be incorporated into a single article. Thus, in one embodiment of the present invention, each AG-nanoarticle is functionalized with two or more recognition elements that possess high affinity to biomolecular targets, the recognition elements being covalently linked to the article polymeric matrix core structure.

Targeting the article could expand the current antibacterial spectrum of aminoglycosides to anaerobic organisms. In particular, isolated ribosomes of most bacterial species are sensitive to aminoglycosides, but transport across the cell membrane is a limiting step. Aerobic bacteria present an oxygen-dependant system that favors the entry of polycationic species, a system that anaerobic bacteria lack. By displaying REs that favor the uptake of the article by other mechanisms, it is possible to target anaerobic bacteria as well.

Recognition elements can be used to direct the aminoglycoside-containing nanoarticle to the site of infection. The recognition element may be chosen from those that target or have binding affinity for either markers of the infected tissue or for the pathogen itself, such as, for example, a particular bacteria, or macrophages, or inflammatory tissue.

The REs can provide enhanced binding to genetic material, either RNA, RNAi or DNA. Such compounds, known to the art, include, but are not limited to, small molecules, intercalating agents, oligonucleotides, peptides, proteins or polycationic polymers known to form complexes with genetic material.

The direct targeting of bacterial pathogens can be achieved with different types of ligands such as, but not limited to, peptides, oligosaccharides, monoclonal antibodies, antibody fragments, and combinations thereof known in the art to bind to or perforate bacteria cell walls. Targeting the AG-nanoarticle to the bacteria allows locating the AG payload in immediate proximity to the bacteria. This creates a high local concentration

without the toxicity associated with a high systemic concentration of AG. In addition, the targeting elements can have a bacteriostatic or antibacterial effect in and of themselves, constituting a two-pronged approach. Their grafting on the NP can even increase such properties either by preventing fast clearance of the small molecules (peptide or short saccharides), by presenting the targeting element at the surface of a bulkier construct (MAb display, steric hindrance enhancing competitive binding), or by a multivalent binding effect. Furthermore, the high water content of the nanoarticle core provides compliancy through a more flexible polymer network. Thus, when attaching to the bacteria surface receptors, the AG-nanoarticles are able to better conform to the curvature of the surface, allowing more surface receptors to be bound. Finally, binding to more receptors may allow the targeted AG-nanoarticle to better function as an antagonist.

Frequently the pathogen is able to survive inside the macrophage, making it harder to achieve successful antibiotic treatment. Studies have shown peptide derivatives of immunomorphin, a sequence of the human IgG H-chain, to be potent stimulators of phagocytosis, enhancing the macrophages' ability to digest virulent strains and non-immunogenic at physiological concentrations. The minimum sequence VKGFY, in cyclic and linear form, stimulates macrophage bactericidal activity through non-opioid p-endorphin receptors (Navolotskaya EV, et al. (2003), *Biochem Biophys Res Comm* **303**, 1065-1072). These peptides bind to macrophages with high affinity. Thus, targeting AG-nanoarticles to the macrophages can enhance efficacy. As for the potential of (oligo)saccharide targeting, various derivatives of L-fucose, D-mannose and L-galactose have been shown to stimulate macrophage migration. The effect was more pronounced when the sugar derivatives were conjugated to BSA, making the migration enhancement factors non-dialyzable (Takata I., et al. (1987), *Journal Leukoc. Biol.* **41**, 248-56). Covering the surface of AG-nanoarticles with these sugars can enhance their macrophage "homing" effect.

The AG-nanoarticle can also target sites in inflammatory tissues, such as receptors over-expressed during inflammation, or fibrin and other indirect manifestations of inflammation analogous to microthrombi. For example, in the first case, peptides comprised of the consensus sequence Arg-Gly-Asp (RGD) can be used to target Integrin $\alpha_V\beta_3$, a well-characterized surface receptor that is over-expressed in inflammatory tissues. The attachment of the target via a tether or linker is of particular importance for RGD targeting, as the peptide docking in the integrin receptor has to access an internal grove of the protein. In the second case, for example, inflammation linked with bacterial infection can manifest itself by the formation of microinjuries analogous to microthrombi. Thus, antifibrin monoclonals, F(ab)' fragments, or peptides, all of which have been produced, can be conjugated to AG-nanoarticles to localize treatment. An AG-nanoarticle targeted with a

peptide that binds to fibrin is expected to densely and specifically adhere to the surface of the microclots, where the bacteria become entangled in the fibrin net. The targeted nanoarticles can create a layer encasing the clot surface, allowing diffusion of the AG in a place that is not reached easily by white blood cells. The targeted AG-nanoarticles would also compete or be positioned near *Pseudomonas aeruginosa* adhering to injured tissue due to infection (e.g. by influenza), burns or scarring (intubation, catheter).

After the assembled building blocks are crosslinked to form the hydrogel scaffold and the aminoglycoside has been incorporated into or attached to the scaffold, the article surface may be functionalized with REs. The REs can be linked either directly or through a linker molecule to the surface of the article.

In a linker configuration, part or all of the REs are "displayed" at the end terminus of the tether or linker. Therefore, in one application of the invention, the articles consist of REs displayed on a hydrogel scaffold. In another embodiment of the invention, the articles consist of an RE, such as a high affinity peptide, linked to the surface of the article core scaffold *via* a linker molecule, the linker comprising preferentially polyethylene glycol (PEG).

For each of these embodiments, it is possible to functionalize the articles utilizing several coupling strategies, varying both the order of addition of the different components and the reactive chemical moieties used for the coupling.

The components may be attached to one another in the following sequences. The hydrogel scaffold is first reacted with a di-functional PEG-containing tether, followed by functionalization of the free terminus of a portion of the PEG chain with a RE. Alternatively, the RE is coupled first to the PEG-containing tether, followed by the attachment of the other PEG terminus to the scaffold.

Several combinations of reactive moieties can be chosen to attach the RE to the tether and to attach the tether to the scaffold. In using a series of orthogonal reaction sets, varying some of the scaffold building blocks and/or tethering arms, it is also possible to attach REs having different molecular structures that bind to different receptors, onto the same article scaffold in well-controlled proportions. Reactions using orthogonal reactive pairs can be done simultaneously or sequentially.

As far as reaction conditions are concerned, it is preferable to functionalize the articles in an aqueous system. The surfactants and the oil phase, residual from the synthesis of the hydrogel scaffold, can be removed through the use (singularly or in combination) of solvent washing, for instance using ethanol to solubilize the surfactant and oil while precipitating the articles; surfactant-adsorbing beads; dialysis; or the use of aqueous systems such as 4M urea. Methods for surfactant removal are known in the art.

The RE must contain a functionality that allows its attachment to the article. Preferentially, although not necessarily, this functionality is one member of a pair of chemoselective reagents selected to aid the coupling reaction. (Lemieux, G., Bertozzi, C., *Trends in Biotechnology*, 1998, *16*, 506-513). For example, when the article surface (and/or linkers grafted to its surface) displays a haloacetal, a peptide RE may be attached through a sulfhydryl moiety. A sulfhydryl moiety in the RE structure can be accomplished through inclusion of a cysteine residue.

Coupling is also possible between a primary amine on the article or the linker terminus and a carboxylic acid on the RE. For example, a carboxylate in the peptide structure can be found either on its terminal amino acid, for linear peptides, or through the inclusion of aspartic or glutamic acid residues. The opposite configuration, where the carboxylic acid is on the article and a primary amine belongs to the peptide, is also easily accessible. Many polymerizable building blocks contain acidic moieties, which are accessible at the surface of the articles after their polymerization. As for poly(amino acid)-based REs, a primary amine function can be found either at its N-terminus (if it is linear) and/or via introduction of a lysine residue.

Another example of reactive chemical pairs consists of the coupling of a sulfhydryl with a haloacetyl or maleimide moiety. The maleimide function can be easily introduced, either on a RE, a linker, or the surface of the articles, by reacting other common functionalities (such as carboxylic acids, amines, thiols or alcohols) with linkers through methods known to one of skill in the art, such as described for example by G. T. Hermanson in *Bioconjugate Techniques*, Academic Press Ed., 1996. In a preferred embodiment, the inclusion of CiBA, or other disulfide-containing building blocks, in the scaffold facilitates the attachment of REs through thiol-reactive moieties. After scaffold formation, reduction of the disulfide linkage in CiBA produces free thiols. Linker molecules containing groups that are reactive with thiol, such as bromoacetamide or maleimide, are added to the reduced AG-containing article to attach the linker to the article scaffold. REs are then added, which react with the free terminus of the linker molecules to give RE-functionalized articles. Alternatively, the RE may be attached to one end of the linker molecule prior to attachment of the linker molecule to the reduced article.

Peptides can also be coupled to the article and/or the tether with a reaction between an amino-oxy function and an aldehyde or ketone moiety. The amino-oxy moiety (either on the articles or in the peptide) can be introduced, starting from other common functionalities (such as amines for example), by a series of transformations known to those skilled in the art. In the same way, aldehyde- or ketone-containing articles and aldehyde-containing peptides are readily synthesized by known methods.

The resulting RE-functionalized, AG-containing articles may be used immediately, may be stored as a liquid solution, or may be lyophilized for long-term storage.

The REs may be any small or large molecular structure that provides the desired binding interaction(s) with the cell surface receptors of the targeted molecule. The number of recognition element moieties per article can range from 2 to about 1000, preferably from 2 to 500, and most preferably from 2 to 100. The articles may optionally further be comprised of more than one type of RE. As used herein, a RE "type" is defined as a specific molecular structure.

In one embodiment of the invention, REs are comprised of peptides. Peptides used as REs according to this invention will generally possess dissociation constants between 10^{-4} and 10^{-9} M or lower. Such REs may be comprised of known peptide ligands. For instance, Phoenix Peptides' peptide ligand-receptor library (http://www.phoenixpeptide.com/Peptidelibrarylist.htm) contains thousands of known peptide ligands to receptors of potential therapeutic value. The peptides may be natural peptides such as, for example, lactams, dalargin and other enkaphalins, endorphins, angiotensin II, gonadotropin releasing hormone, melanocyte-stimulating hormone, thrombin receptor fragment, myelin, and antigenic peptides. Peptide building blocks useful in this invention may be discovered via high throughput screening of peptide libraries (e.g. phage display libraries or libraries of linear sequences displayed on beads) to a protein of interest. Such screening methods are known in the art (for example, see C.F. Barbas, D. R. Burton, J. K. Scott, G. J. Silverman, *Phage Display*, 2001, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The high affinity peptides may be comprised of naturally occurring amino acids, modified amino acids or completely synthetic amino acids. The length of the recognition portion of the peptide can vary from about 3 to about 100 amino acids. Preferably, the recognition portion of the peptide ranges from about 3 to about 15 amino acids, and more preferably from 3 to 10 amino acids. Shorter sequences are preferred because peptides of less than 15 amino acids may be less immunogenic compared to longer peptide sequences. Small peptides have the additional advantage that their libraries can be rapidly screened. Also, they may be more easily synthesized using solid-state techniques.

REs may comprise a variety of other molecular structures, including antibodies, antibody fragments (humanized or fully human antibodies, and humanized or fully human antibody fragments), lectins, nucleic acids, and other receptor ligands.

Additionally, it will be possible to design other non-protein compounds to be employed as the binding moiety, using techniques known to those working in the area of drug design. Such methods include, but are not limited to, self-consistent field (SCF)

analysis, configuration interaction (CI) analysis, and normal mode dynamics computer programs, all of which are well described in the scientific literature. See Rein et al., *Computer-Assisted Modeling of Receptor-Ligand Interactions*, Alan Liss, New York (1989).

In particular, in one other embodiment of the invention, the REs are composed of carbohydrates and carbohydrate derivatives, such as, but not limited to sialic acid, galabiose, mannose, lactose, galactose, sequence-specific trioses, tetraoses, pentoses, hexoses, glycans, amino or acetylamino-carbohydrates and their phosphorylated derivatives. In many cases, interaction of carbohydrates with carbohydrate-binding proteins (lectins, adhesins, siglecs, selectins, collectins and enzymes of carbohydrate metabolism) may be used for specific drug targeting to specific mammaliancells or organs. In other cases, interaction of carbohydrates with cell surface receptors and biological membranes may provide specific targeting toward the infectious micro-organism. Preparation of non-protein compounds and moieties will depend on their structure and other characteristics and may normally be achieved by standard chemical synthesis techniques. See, for example, *Methods in Carbohydrate Chemistry*, Vols. I-VII, and *Analysis and Preparation of Sugars*, Whistler et al., Eds., Academic Press, Inc., Orlando (1962), the disclosures of which are incorporated herein by reference.

The use of multiple RE molecules of the same molecular structure or of different molecular structure to make up the article can increase the avidity of the article. As used in the present invention, "high affinity" means a binding of a single RE to a single target molecule with a binding constant stronger than 10^{-4} M, while "avidity" means the binding of two or more such RE units to two or more target molecules on a bacteria, a host cell or molecular complex.

Difficulties in penetrating or translocating across microbial cell membranes put a severe limitation on the therapeutic efficiency of aminoglycosides. REs playing the role of carrier peptides facilitate mediated delivery of the nanoarticles into the cell or the microorganism. For example, peptides comprised of the amino acid sequence VLTNENPFSDP have been shown to facilitate delivery into *Candida albicans* and *Staphylococcus aureus*, peptides comprised of the amino acid sequence YKKSNNPFSD facilitate delivery into *Bacillus subtilis*, while those comprised of the sequence CFFKDEL facilitate delivery into *Escherichia coli* (Karolinska Instituet, FEMS Microbiol. Lett., vol. *215*, 2002, p 267-72).

The REs can also target a multitude of disease-associated biomolecules. Tumor-associated targets include erbB1 (for example, using as REs the growth factor EGF, or using peptides comprised of the amino acid sequence YCPIWKFPDEECY, or other sequences found in Greene, et.al. J. Biol. Chem., 2002, *277(31)*, 28330-28339), erbB2 (for

example, using as REs peptides comprised of the amino acid sequence CdFCDGFdYACYMDV (where dF and dY representing the D isomer of the amino acid residues) or other sequences delineated in Murali, J. Med. Chem., 2001, *44*, 2565 - 2574), erbB3, erbB4, CMET, CEA (for example, using peptides disclosed in PCT WO 01/74849 as REs), and EphA2. Vascular targets associated with multiple pathologies, including inflammation and cancer, include VEGFR-1, VEGFR-2 (for example, using as REs peptides comprised of the amino acid sequence ATWLPPR (as described in Demangel, et.al. , EMBO J., 2000, *19(7)*, 1525-1533), integrins (including integrin $\alpha v\beta 3$ and integrin $\alpha v\beta 1$), and to extracellular proteins such as fibrin (which may be targeted using peptides comprised of amino acid sequences disclosed in PCT Application WO 02/055544).

The following non-limiting Examples are provided to further describe how the invention may be practiced.

EXAMPLES

Example 1: IMMA (inulin multi-methacrylate) synthesis

Inulin (4 g) was weighed into a 1 neck round bottom flask. A Teflon-coated stir bar was added and the flask was sealed with a septa. Anhydrous pyridine (approximately 20 mL for each 5 grams of inulin) was then transferred into the flask, keeping the entire system under a blanket of nitrogen. The mixture was stirred until the inulin dissolved, then 1 mL of methacrylic anhydride was added dropwise, using a syringe. The reaction was allowed to continue for 16 hours. After that interval, enough pyridine was removed under vacuum to make a viscous liquid. Toluene (approximately 40 mL) was then added with vigorous mixing to precipitate the crude product. The liquid was then decanted from the precipitate. The solid was dissolved in water, producing a viscous, but free flowing, syrup, which was then precipitated with 2-propanol (approximately 200 mL). This process of water dissolution followed by precipitation was repeated twice more, after which the product was dried under vacuum. The product was then redissolved in 50 mL of water and filtered through a filter paper to remove accumulated dust and other insolubles, and the product was then lyophilized to give inulin multi-methacrylate (IMMA). IMMA identity (degree of methacrylate functionalization) was confirmed by NMR analysis.

Example 2: CiBA synthesis

Sodium hydroxide, (2.0 g) was dissolved in 70 mL of dry methanol. L-cystine (2.73 g) was added to the methanolic NaOH solution, and the round bottom flask containing the mixture was immersed in an ice water bath to maintain the reaction vessel at zero degrees

Celsius. Acryloyl chloride (2.22 mL) was then added to the methanolic cystine solution dropwise.

The reaction was covered and stirred for 1 hour at RT, after which the reaction solution was centrifuged and the liquid phase was decanted off into rapidly stirred ethyl acetate (120 mL). The resulting suspended solids were isolated by centrifugation and were dried under vacuum. The identity of the isolated material was confirmed by ^{1}H NMR as N,N'-cystinebisacrylamide (CiBA).

Example 3: PEG-1500 dBA synthesis

Poly(ethylene glycol) (average molecular weight = 1500, 15.36 g) was dissolved in 75 mL of dry chloroform contained in a round bottom flask. A stir bar was added to aid in the dissolution process and to maintain reaction homogeneity. Bromoacetyl chloride (4.00 mL) was added, an air-cooled reflux condenser was attached to the round bottom flask, and the reaction mixture was heated at reflux under a nitrogen purge vented to the atmosphere (to remove HCl gas generated during the reaction). After 4 hours, more bromoacetyl chloride (1.0 mL) was added and the reaction mixture was heated for an additional 5 hours. The reaction mixture was cooled and stirred gently overnight. The following day, the solvent and excess reagent were removed under vacuum, and the residue was dissolved in saturated sodium bicarbonate. The water solution was extracted with chloroform (4 times 50 mL). The organic extractions were combined, dried over magnesium sulfate, and were filtered. Removal of the solvent under vacuum left PEG-1500 dBA as an off white solid. The identity of the product was confirmed by ^{1}H NMR.

Example 4: Preparation of heterobifunctional PEG-400, bromoacetate and carboxylic acid

Poly(ethylene glycol) dibromoacetate (molecular weight average 400, PEG-400 dBA) (4.0 g) was dissolved in 150 mL pH=8, phosphate buffer (0.15 M) containing 100 mL THF. To this solution, 3-mercaptopropionic acid (0.17 g) in water (10 mL) was added with rapid mixing provided by a stir bar. The pH was adjusted to 8 again by the addition of a 1.0 M NaOH solution. Sixteen hours after the addition, the volume of the reaction was reduced under vacuum to 50 mL. The volume was increased to 150 mL by adding 100 mL pH=8.0 phosphate buffer (0.10 M) and the water solution was extracted with chloroform (2 times 50 mL) to remove unreacted PEG starting material. The pH of the solution was adjusted to 2 by adding 1.0 M HCl, and the solution was extracted again with chloroform (3 times 50 mL). The combined extracts of the pH=2.0 solution were dried with sodium sulfate and filtered. Removal of the solvent under vacuum yielded the target compound.

Example 5: IMMA-CiBA-NaA-Gentamicin acrylate

(Polymerizable Gentamicin salts, free radical polymerization)

Gentamicin sulfate was passed through an ion exchange column (BioRad AG 1X2) previously loaded with 25 vol% acrylic acid solution in water till the eluant pH< 4.8. The fractions were collected and the resulting gentamicin acrylate product was lyophilized.

An aqueous phase was prepared by combining 77 wt% water, 14 wt% IMMA, 2 wt % CiBA , 1 wt% sodium acrylate and 6 wt% gentamicin acrylate. An oil+surfactant phase was prepared by mixing Igepal CO-210, Igepal CO-720 and cyclohexane in a weight ratio of 1.0:1.3:9.0. Three grams (3 g) of the aqueous phase were mixed with 30 g of the oil+surfactant phase, resulting in the formation of a reverse microemulsion. The reverse microemulsion contained surfactant-stabilized nano-droplets of aqueous phase dispersed in a continuous phase of cyclohexane. To the reverse microemulsion was added an aqueous solution containing Eosin Y, where the photoinitiator represented from 0.001 to 0.1 wt% of the monomers mass. The reverse microemulsion was degassed with freeze-thawing cycles under vacuum, with N_2 gas backfill between cycles. The contents were stirred and irradiated with a UV or visible light source of at least 100 W for 20 min to two hours to polymerize the building blocks. Once the polymerization was completed, the nanoarticles were precipitated by adding 9 mL of pure ethanol directly to the solution. The nanoarticle-containing pellets were resuspended in water. Residual surfactants and solvents were removed by standard techniques (dialysis, chromatography, etc.). At this point, the aqueous solution of nanoarticles was lyophilized.

Example 6: Schiff base pre-assembly, free radical polymerization

IMMA-CiBA-DAA-Gentamicin:

An aqueous phase was prepared by combining 75.4 wt% PBS, pH 7.2, 14 wt% IMMA, 2 wt % CiBA, 6.6 wt% diacetone acrylamide (DAA) and 4 wt% gentamicin sulfate. The aqueous phase was warmed up to 50°C for an hour. An oil+surfactant phase was prepared by mixing Igepal CO-210, Igepal CO-720 and cyclohexane in a weight ratio of 1.0:1.3:9.0. Three grams (3g) of the aqueous phase were mixed with 30 g of the oil+surfactant phase, resulting in the formation of a reverse microemulsion. The reverse microemulsion contained surfactant-stabilized nano-droplets of aqueous phase dispersed in a continuous phase of cyclohexane. To the reverse microemulsion was added an aqueous solution containing Eosin Y, where the photoinitiator represented from 0.001 to 0.1 wt% of the monomers mass. The reverse microemulsion was degassed with freeze-thawing cycles under vacuum, with N_2 gas backfill between cycles. The contents were stirred and irradiated

with a UV or visible light source of at least 100 W for 20 min to two hours to polymerize the building blocks. Once the polymerization was completed, the nanoarticles were precipitated by adding 9 mL of pure ethanol directly to the solution. The nanoarticle-containing pellets were resuspended in water. Residual surfactants and solvents were removed by standard techniques (dialysis, chromatography, etc.). At this point, the aqueous solution of nanoarticles may be lyophilized, if desired.

ELISA tests for gentamicin showed that approximately 50 wt% of gentamicin was accessible to antibodies in pH 7.2 PBS buffer.

Representative examples of nanoarticles fabricated following the above procedure are listed below, with the building blocks wt % composition of their respective aqueous phases in the reverse microemulsion (remaining weight of the aqueous phase is water):

wt% IMMA	wt% CilBA	wt% DAA	wt% Aminoglycoside
36	2	2	4 kanamycin
28	4	2	1.2 gentamycin
28	2	2	1.2 gentamycin
28	2	1	3.3 tobramycin
14	2	6.6	4 gentamycin
14	2	3	7.4 gentamycin
14	2	3	1.82 gentamycin
14	2	1	7.4 gentamycin
14	2	1	0.55 gentamycin
14	0	6.6	4 gentamycin
14	0	1	1.65 tobramycin

Nanoarticles have also been likewise synthesized following the above procedure with compositions containing other building blocks such as, for example, methylene bis acrylamide (MBA) or tetraethyleneglycol diacrylate (TEGA), representative examples of which are:

14/2/1/7.4 IMMA/MBA/DAA/Tobramycin

28/2/1/3.3 IMMA/TEGA/DAA/Tobramycin

Example 7: IMMA-CiBA-NaA-Gentamicin

(free radical polymerization followed by amide bond formation)

An aqueous phase was prepared by combining 72 wt% PBS, pH 7.2, 25 wt% IMMA, 2 wt % CiBA, and 1 wt% sodium acrylate (NaA). An oil+surfactant phase was prepared by mixing Igepal CO-210, Igepal CO-720 and cyclohexane in a weight ratio of 1.0:1.3:9.0 and

warmed up at 50°C for an hour. Three grams (3 g) of the aqueous phase were mixed with 30 g of the oil+surfactant phase, resulting in the formation of a reverse microemulsion. The reverse microemulsion contained surfactant-stabilized nano-droplets of aqueous phase dispersed in a continuous phase of cyclohexane. To the reverse microemulsion was added an aqueous solution containing sodium persulfate as thermal initiator and TEMED as accelerator. The reverse microemulsion was degassed with freeze-thawing cycles under vacuum, with N_2 gas backfill between cycles. The contents were stirred at room temperature for an hour to 24 hours to polymerize the building blocks. Once the polymerization was completed, the nanoarticles were precipitated by adding 9 mL of pure ethanol directly to the solution. The nanoarticle-containing pellets were resuspended in water. Residual surfactants and solvents were removed by standard techniques (dialysis, chromatography, etc.). At this point, the aqueous solution of nanoarticles may be concentrated further by methods known to the art, such as by cross-flow dialysis or lyophilized, if desired.

To an aqueous solution of the previously prepared nanoarticles at 50 mg/mL in 0.1 M HEPES, pH 7.5, are added 1.1 eq moles N-hydroxysuccinimide and 0.213 moles of EDC per mole of total acid available in the nanoarticle core. The activating reaction is allowed to proceed for ca 10 min and followed by addition of 1/4th of the final desired tobramycin quantity (tobramycin is added also as solution in a pH 7.5 buffer). The previous step is repeated three more times, keeping an interval of 20 min between each repeat. Unreacted reagents are removed by any appropriate method for the quantity synthesized (flow through dialysis, column, spin-filtration, etc). The reaction ratio of aminoglycoside over available acid moieties in the nanoarticle core can be varied, preferentially between 0.25 to 2 amine function per acid moiety.

MIC value for such a nanoarticle synthetized containing 15% tobramycin was similar to the one of free tobramycin (9.5 µg/mL vs 8 µg/mL)

Example 8: Scaffold formation via reaction of oxidized inulin (*in-situ*) with gentamicin and carbohydrazide

An aqueous phase was prepared by combining 90 wt% 0.2 M borate KCl buffer pH 8.5 and 10 wt% inulin. An oil+surfactant phase was prepared by mixing Igepal CO-210, Igepal CO-720 and cyclohexane in a weight ratio of 1.0:1.3:9.0. Three grams (3 g) of the aqueous phase were mixed with 40 g of the oil+surfactant phase, resulting in the formation of a reverse microemulsion. One gram of a sodium periodate solution in water was added to the mixture, so that there was a maximum of one periodate equivalent per glucose monomer unit (from the inulin). The in-situ oxidation proceeded for 5 to 20 minutes. A concentrated solution of gentamicin in borate buffer was added (so that final concentration

in aqueous phase equals 5 wt%) and the reaction was allowed to proceed for 4 hours at 50°C. A concentrated solution (of at least 1g/mL) of carbohydrazide in buffer (50 to 250 mM, pH 8 to 9) was then added to the microemulsion and allowed to react overnight at 4°C. The resulting nanoarticles obtained by the crosslinking of the aldehydes functionalities of oxidized dextran with the aminoglycosides (to form Schiff bases) and the carbohydrazide (to form carbohydrazones) were isolated by precipitation in the presence of ethanol and centrifugation, and purified from the excess of unreacted reagents by dialysis.

Example 9: Scaffold formation via reaction of oxidized dextran and glutaraldehyde with gentamicin

An aqueous phase was prepared by combining 90 wt% 0.2 M borate KCl buffer pH 8.5 and 10 wt% oxidized dextran. An oil+surfactant phase was prepared by mixing Igepal CO-210, Igepal CO-720 and cyclohexane in a weight ratio of 1.0:1.3:9.0. Three grams (3 g) of the aqueous phase were mixed with 40 g of the oil+surfactant phase, resulting in the formation of a reverse microemulsion. A concentrated solution of gentamicin in borate buffer was added (so that final concentration in aqueous phase equals 5 wt%) and the reaction was allowed to proceed for 4 hours at 50°C. A concentrated solution of glutaraldehyde (50 wt% in water) was then added dropwise to the microemulsion (so that the final concentration of glutaraldehyde in aqueous phase equals 1 wt%) and allowed to react for 15 min. The resulting nanoarticles obtained by the crosslinking of the aldehydes functionalities of oxidized dextran and glutaraldehyde with the aminoglycosides (to form Schiff bases) were isolated by precipitation in the presence of ethanol and centrifugation, and purified from the excess of unreacted reagents by dialysis.

Example 10: Scaffold formation via formation of amide and ester bonds

An oil+surfactant phase was prepared by mixing Igepal CO-210, Igepal CO-720 and cyclohexane in a weight ratio of 1.0:1.3:9.0 and warmed up at 50°C for an hour prior to use to ensure homogeneity.

Three grams (3 g) of an aqueous phase was prepared by dissolving an acid-containing oligosaccharide, such as colominic acid, and N-hydroxysuccinimide (1 mole eq. per acid function available) in 0.1 M HEPES, pH 7.5, at 25 wt% and added to 15 g of the oil+surfactant phase, resulting in the formation of a reverse microemulsion A. Two other microemulsions were prepared, one containing concentrated EDC in its aqueous phase, the other one the aminoglycoside at ca 30 wt%.

To microemulsion A containing colominic acid and NHS was added 1/4th of the final volume of the EDC-containing microemulsion. Mixing of the two emulsions and subsequent

carboxylate activation was allowed to proceed for 10 min after which a 1/4th of the final volume of the aminoglycoside containing microemulsion was added and allowed to react 30 min. The serial addition was repeated 3 more times. After completion of the serial additions, the reaction mixture is left to stir overnight at RT. The final ratio in the aqueous phase have been varied from 10 to 20 wt% colominic acid, 0.5 to 10wt% aminoglycoside (gentamycin and tobramycin) and ratios of 1.1 mol eq EDC added per amine moiety on the aminoglycoside.

Further crosslinking can be provided by the optional addition of diacyl chloride to the reverse microemulsion. Some of those acyl chloride functions hydrolyze when entering in contact with the aqueous phase and provide free carboxylates attached by one terminus to the nanoarticle, generating desirable negative charge and/or attachement points for further derivatization (such as targeting). The final quantity of diacyl chloride can amount up to 20 wt% of the final aqueous phase. The nanoarticles are purified from hydrolysed and unreacted diacyl chlorides and other impurities by methods known by those skilled in the art.

Particles may be prepared using the above procedure with the following diacyl chlorides: adipoyl dichloride, succinyl dichloride, malonyl dichloride and diglycolyc dichloride.

Following the previous protocol, nanoarticles may be prepared where colominic acid has been replaced by multi-inulin-sodium succinate and sialic acid.

Example 11: Nanoarticle with a peptide targeting bacteria as RE

A peptide known to inhibit *Pseudomonas aeruginosa* binding has been extended with a cysteine on the N-terminus to allow convenient attachment via a thiol reactive linker. This peptide is constituted of a soluble portion of the CFTR extracellular domain, preferentially containing the sequence SYDPDNKEER.

Nanoarticles containing CiBA, such as those described in Example 6: or Example 7: are reduced in the presence of diotheitol (DTT) to expose free thiols. The solution is quickly scrubbed of unreacted DTT by methods known of those skilled in the art and allowed to react with an excess of PEG dimaleimide for 30 minutes to overnight. The optimal reaction time is proportional to the length of the di-maleimide-PEG. After an hour reaction with PEG-3400 dimaleimide, the cysteine terminated peptide is added to the reaction mixture, allowed to react several hours. The functionalized nanoarticles are purified by appropriate methods (such as flow through dialysis or ethanol precipitation followed by centrifugation) and can be further lyophilized.

Example 12: Nanoarticle with a peptide RE targeting bacteria:

A known amount of cysteine-terminated peptide, such as CSYDPDNKEER, is dissolved into 0.5 M PBS pH 7.2 at 20 mg/mL, to which is added 1 equivalent of a sulfhydryl-reactive and carbonyl-reactive heterobifunctional cross-linking agent, such as EMCH. The reaction is allowed to proceed for 2 hours, after which pH is adjusted to 5 using 1M HCl. Nanoarticles containing aldehydes such as those described in Example 8: and Example 10: or ketones such as DAA in nanoarticles described in Example 6: are dissolved in 100 mM phosphate buffer pH 5 to a concentration of 20 mg/mL. The peptide-linked EMCH is added, up to 1 equivalent of linker for each mole of carbonyl contained in the nanoarticle, and allowed to react for 20 hours at 37°C. Immediately upon addition of the two solutions, the pH is adjusted to 5 by addition of the sufficient quantity of 100 mM phosphate buffer pH 5.

Example 13: Nanoarticle with a peptide RE targeting macrophages:

A similar method as in Example 12: has been used to synthesize nanoarticles targeting macrophages.

Studies have shown peptide derivatives of immunomorphin, a sequence of the human IgG H-chain, to be potent stimulators of phagocytosis, enhancing the macrophages' ability to digest virulent strains and non-immunogenic at physiological concentrations. The peptide contains preferentially the minimum sequence VKGFY, in cyclic and linear form, which stimulates macrophage bactericidal activity through non-opioid p-endorphin receptors.

For example, nanoarticles synthesized as described in Example 6:, containing 25/2/1 IMMA/CIBA/DAA and 1.65 tobramycin have been targeted with a longer linear sequence from the same IgG chain, CLVKGFY, via an EMCH linker. The resulting nanoarticles showed increased *in vitro* and *in vivo* activity over non-targeted nanoarticles of equivalent AG concentration.

Example 14: Nanoarticle with a peptide RE with antibacterial properties

FQWQRWMRKVR is a multiple antigen peptide homologous to just over half the loop region of human lactoferrin that showed antibacterial activity against a broad spectrum of bacteria, especially strong against *P. aeruginosa* . This peptide is extended by a glycine protected by benzoyl benzoic acid during the solid phase synthesis.

The peptide is dissolved in a solution of nanoarticles which present free amine functionalities (preferentially containing a high ratio of aminoglycoside over other reactive moieties). The peptide attachment proceeds by activation of the benzoyl group by UV irradiation at 4°C.

Example 15: In vitro activity of gentamicin-nanoarticles

The minimal inhibition concentrations (MIC) of gentamicin-nanoarticles and free gentamicin were tested with the following bacteria strains:

E. coli (AG100, Stuart Levy's standard ref. K12 strain) (gram-negative)

E. faecalis (Patrice Courvalin's reference strain) (gram-positive)

P. aeruginosa (PA01, reference strain) (gram-negative)

The MIC was determined by the standard broth dilution technique. The tube dilution test is the standard method for determining levels of resistance to an antibiotic. Serial dilutions of the antibiotic are made in a liquid medium, which is inoculated with a standardized number of organisms and incubated for a prescribed time. The lowest concentration (highest dilution) of antibiotic preventing appearance of turbidity is considered to be the minimal inhibitory concentration (MIC). At this dilution the antibiotic is bacteriostatic.

MIC	Free gentamicin	Articles from Examples 6 and 7
Escherichia coli	12.5 μg/mL to 15 μg/ml	50 μg articles/mL to 60 μg articles/mL (AG per NP needed)
Enterococcus faecalis	125 μg/mL	250 μg articles/mL
Pseudomonas aeruginosa	8 μg/mL	62 μg articles/mL

Based on the formulation composition, which had a loading of up to 16 % of tobramycin the above results show that the gentamicin-containing articles are more efficient than pure gentamicin. TEM and DLS showed that gentamicin-containing articles are in 30-50 nm diameter range.

Example 16: In vitro activity of tobramycin-nanoarticles

The minimal inhibition concentrations (MIC) of tobramycin-nanoarticles made by the same procedure as gentamicin-nanoarticles described in Examples 6 & 7, and free tobramycin were tested with *Pseudomonas aeruginosa* (PA01).

The MIC was determined by the standard broth dilution technique as described in Example 15.

MIC	Free tobramycin	Tobramycin-nanoarticles
Pseudomonas aeruginosa	8 μg/mL	20 μg articles/mL to 62 μg articles/mL

Based on the formulation composition, which had a loading of up to 20% of tobramycin, the above results show that the tobramycin-containing articles are more efficient than pure tobramycin.

Example 17: Time-dependent inhibition in bacterial proliferation by free tobramycin and tobramycin-nanoarticles.

The inhibition of proliferation of cultures of *P. aeruginosa* in exponential growth stage (log phase) when incubated with free tobramycin and tobramycin-nanoarticles such as used in Example 16 was almost identical. Only a 30-minute delay in the complete inhibition of proliferation was observed compared to free tobramycin, which can be explained by the slow release of tobramycin from the nanoarticle core. The result suggests that enzymes secreted by *P. aeruginosa* were fast to detach tobramycin from the nanoarticle.

Example 18: In vivo activity of tobramycin-nanoarticles

Mice were intravenously injected with free tobramycin, tobramycin-nanoarticles and saline (negative control) 30 minutes before intratracheal instillation with 50 μL of 1.46×10^7 colony-forming units/mL of *Pseudomonas aeruginosa*. Nanoarticles were synthesized as described in Example 6 containing 14/2/1 IMMA/CiBA/DAA and 1.65 trobramycin. Targeted nanoparticles were made as described in Example 13 containing 14/2/1/ IMAA/CiBA/DAA and 1.65 tobramycin and functionalized with the linear sequence CLVKGFV. The following groups were used (n=3):

Group	Treatment	Dose
1	free Tobramycin	18 mg/kg
2	Tobramycin-nanoarticle	200 mg/kg (equiv. to 18 mg tobramycin)
3	Targeted tobramycin-nanoarticle	200 mg/kg (equiv. to 18 mg tobramycin)
4	Saline	

During the treatment, the body temperature of the animals was measured (Table 2). At the end of the treatment period, mice were euthanized, and the lungs dissected and homogenized to determine the number of colony-forming units per lung (Table 1). With tobramycin-nanoarticles (NP) and targeted tobramycin-nanoarticles (NP-targeted) there was a larger reduction in the number of bacteria in the lungs than among animals treated with free tobramycin. The group that was treated with targeted tobramycin-nanoarticles showed the most significant reduction in bacterial cell count, with one animal showing complete eradication of bacteria in the lungs. The body temperature of animals treated with free

tobramycin or tobramycin-nanoarticles dropped slightly during the first 6 hours post instillation with bacteria and returned back to almost normal body temperature within 12 hours (Table 2). The body temperature of animals treated with targeted tobramycin-nanoarticles dropped significantly during the first 6 hours post instillation with bacteria but returned back to almost normal body temperature within 12 hours, while animals treated with saline showed the biggest drop in temperature and the body temperature did not return to normal levels within 48 hours.

Table 1: Bacteriology

Group	No	Treatment and dose	CFU/lung
1	1	TOB 18 mg/kg iv	28000
	2	TOB 18 mg/kg iv	290
	3	TOB 18 mg/kg iv	70
	Average		9453.3
	SD		16062.3
2	4	NP 200 mg/kg iv	70
	5	NP 200 mg/kg iv	80
	6	NP 200 mg/kg iv	390
	Average		180
	SD		181.9
3	7	NP-targeted200 mg/kg iv	10
	8	NP-targeted200 mg/kg iv	410
	9	NP-targeted200 mg/kg	0
	Average		140.0
	SD		233.9
4	10	Saline iv	Died
	11	Saline iv	Died
	12	Saline iv	>100,000
	Average		
	SD		

Table2 : animal body temperatures.

group	hrs	0	1	2	3	4	5	6	8	12	25	36	48
1	Av	38.1	36.8	35.5	34.7	34.8	36.2	36.3	36.3	37.6	36.3	36.5	35.9
	SD	0.25	0.71	0.2	0.55	0.85	0.15	0.15	0.36	0.4	0.58	0.44	0.72
2	Av	37.9	36.8	35.6	34.6	35.6	35.8	36.6	36.5	37.5	36.1	36.9	35.6
	SD	0.31	0.38	0.47	0.7	1.4	1.11	0.2	0.44	0.25	0.15	0.15	0.47
3	Av	38.7	36.9	34.3	31.8	30.2	30.9	32.4	35	36.1	36.4	37.2	36.3
	SD	0.91	0.4	0.2	0.75	1.97	2.41	2.7	1.32	0.5	0.1	0.38	0.21
4	Av	38.6	36.4	31.1	29.9	28.5	28.2	28.3	29.1	31.2	33.7	33.3	33.4
	SD	0.5	0.31	1.29	1.21	0.45	1.01	0.42	1.36	0.96	0	2.4	

Example 19: In vivo nephrotoxicity study of tobramycin-nanoarticles

Twenty male Sprague-Dawley rats were assigned to five treatment groups (4 tests and one control) and administered a single daily intravenous dose over a period of seven consecutive days of tobramycin-nanoarticles, free tobramycin, or PBS (negative control). Nanoarticles were synthesized according to Example 6:. The test and control articles were administered by tail vein injection on a mg/kg weight basis. Clinical observations were performed prior to dose administration, following dosing and daily thereafter to assess survival and general conditions. Blood was collected daily from animals prior to dosing for clinical chemistry and tobramycin evaluation. The following clinical chemistry parameters were measured. If there was not enough serum for analysis of all parameters, the serum was diluted and assays were performed according to priority in the order listed below: BUN, creatinine, total protein, albumin, sodium, potassium, chloride, calcium and phosphorus. Urine was collected from all animals prior to the final blood collection on day 7. Animals were anesthetized using CO_2 followed by terminal exsanguinations from vena cava on day 7 and subjected to gross necropsy. A gross pathological examination was performed and the kidneys were collected for histological evaluation and fixed in 10% formalin. Animals were weighed on first and last day and their food consumption measured quantitatively. Organs were weighed on last day after a dry pat, right and left kidneys were weighed separately and relative tissue weights were calculated.

The four treatment groups were as follow:

Tobramycin-nanoarticles	564 mg/kg (eq to 50 mg/kg tobramycin)
Tobramycin-nanoarticles	282 mg/kg (eq to 525 mg/kg tobramycin)
Tobramycin	50 mg/kg
Tobramycin	25 mg/kg
PBS	Same volume as above (5 mL/kg)

All animals consumed a similar amount of food and gained a similar amount of weight over the course of the study.

In conclusion, no significant evidence of nephrotoxicity was observed in the Sprague Dawley rats administered a single daily intravenous dose of 282 mg/kg and 564 mg/kg of tobramycin nanoarticles for 7 days.